be separated with considerable purity from complexed biological mixtures with Dowex-50 (NH_4^+) and elution with dilute ammonia[7]. This is the preferred method for the isolation and purification of this arginine analogue. Unfortunately, histidine is one of the very few natural products that co-elutes with canavanine under these experimental conditions. Given the instances of erroneous disclosure of canavanine's natural occurrence and the potentially universal distribution of free histidine, it is evident that assertions on canavanine's occurrence and distribution must not be predicated solely on the use of PCAF or the elution position relative to canavanine of a given natural product.

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Purine nucleotide cycle as a possible anaplerotic process in rat skeletal muscle[1]

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Summary. The intermediates of the purine nucleotide cycle (PNC) stimulated pyruvate oxidation by isolated skeletal muscle mitochondria in a system containing mitochondria and cytosol from rat skeletal muscle. Thus, in skeletal muscle the PNC might be involved in the anaplerotic supply of tricarboxylic acid cycle intermediates.

Three reactions have been suggested which might cause an increase in the content of citric acid cycle intermediates in skeletal muscle[2-6]. These reactions are: a) pryruvate carboxylation catalyzed by pyruvate: carbon dioxide ligase (ADP-forming), (EC 6.4.1.1.)[3,4], b) malate formation catalyzed by extramitochondrial malic enzyme (l-malate: $NADP^+$ oxidoreductase oxaloacetate decarboxylating), (EC 1.1.1.40)[5], c) the reactions of the PNC[2,6]. Some authors have also taken into consideration alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase), (EC 2.6.1.2) and glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase deaminating), (EC 1.4.1.2)[2,6]. However, a major role for the last 2 enzymes seems unlikely[6]. The role of pyruvate carboxylase[4] and extramitochondrial malic enzyme[5] in replenishing the Krebs cycle intermediates in skeletal muscle have gained strong support from the recent experiments performed on isolated mitochondria. Aragon and Lowenstein[6] showed that in rat skeletal muscle the total level both of citric acid cycle intermediates and of IMP rises during exercise. They suggest therefore that the PNC is operating in skeletal muscle during exercise and that it might be responsible for an increase of citric acid cycle intermediates.

In this communication, a system is described in which fumarate formed during operation of the purine nucleotide cycle in skeletal muscle cytosol stimulated pyruvate oxidation in isolated muscle mitochondria. The results gave further evidence that the operation of the purine nucleotide cycle may be responsible for the replenishment of citric acid cycle intermediates in skeletal muscle.

Materials and methods. Pyruvate, malate, fumarate, adenylosuccinate, aspartate, GTP, AMP and IMP were from Sigma Chem. Co (USA). All other chemicals were from P.O.Ch. Gliwice (Poland).

Skeletal muscle mitochondria were prepared as described previously[7]. The muscle cytosol was prepared from the hind legs of rats. The muscles were homogenized in 3 volumes of 50 mM phosphate buffer pH 7.0 containing 15 mM KCl and 1 mM dithiothreitol and centrifuged at 20,000 × g for 40 min. The pellet was rehomogenized, and centrifuged again at 20,000 × g for 40 min. The supernatants were combined and centrifuged at 100,000 × g for 40 min. The resulting supernatant was dialyzed overnight against 500 vols of homogenization medium without dithiothreitol. Ammonia was determined according to the method of Seligson and Seligson[8] as modified by Strelkov[9]. After microdiffusion, ammonia was measured colorimetrically by the method of Chaney and Marbach[10]. Oxygen consumption was measured with a Clark electrode under the conditions indicated in the appropriate tables and figures.

Results and discussion. The isolated rat skeletal muscle mitochondria were able to oxidize pyruvate at a low rate in the absence of added malate. When malate was added, the oxygen uptake increased several-fold due to the entry of pyruvate into the citric acid cycle. Fumarate could replace malate in such experiments. Figure 1 demonstrates that in the mitochondrial suspension oxidizing pyruvate, the rate of oxygen uptake increased from a very low rate (about 16 ng-atoms $O \times min^{-1} \times mg^{-1}$) to a maximum rate of about 280 ng-atoms $O \times min^{-1} \times mg^{-1}$ while the concentration of either L-malate or fumarate was increasing. The concentration of L-malate required to reach the maximum rate of oxygen consumption was about 0.5 mM. Similar results were obtained when malate was replaced by fumarate. A possible explanation is that fumarate is taken up into the mitochondria where it is converted to malate by intramitochondrial fumarase. This might be somewhat surprising, because fumarate is claimed to be a substrate that is unable to penetrate into rat liver mitochondria[11]. However, some evidence that fumarate translocation in rat heart mitochondria does take place has been presented recently[12]. It seems likely that this is the case in rat skeletal muscle mitochondria also. However, it is not excluded that the conversion of fumarate to malate is taking place outside the

inner mitochondrial membrane, catalyzed by the extramitochondrial fumarase bound to the mitochondrial membrane.

The fact that fumarate is able to stimulate oxygen consumption in mitochondria oxidizing pyruvate prompted us to study the effect on pyruvate oxidation by isolated rat skeletal mitochondria of the fumarate formed during the operation of the PNC according to the net reaction: aspartate + GTP + H_2O → NH_3 + fumarate + GDP + P_i. In the 1st reconstruction experiment shown in figure 2, it was observed that the rat skeletal muscle mitochondria oxidized added pyruvate at a low rate. Addition of adenylosuccinate to the incubation medium did not change oxygen consumption. A further addition of cytosol markedly stimulated the respiration. The maximum rate of respiration under these conditions amounted to about half of the rate obtained with pyruvate plus fumarate (see fig. 1). The addition of cytosol to the mitochondria oxidizing pyruvate without added adenylosuccinate had no effect on oxygen consumption. This indicates that a high rate of respiration was obtained only when both adenylosuccinate and cytosol were added. The only reasonable explanation we can offer for the events shown in figure 2 is that the added adenylosuccinate was converted to fumarate by the adenylosuccinate lyase present in rat muscle cytosol, according to the reaction: adenylosuccinate → AMP + fumarate. The resulting fumarate stimulated pyruvate oxidation, as shown in figure 1.

In order to check whether, in fact, the operation of the purine nucleotide cycle is replenishing Krebs cycle intermediates by producing fumarate, the experiments presented in the table were carried out. The addition of IMP, GTP and aspartate to the incubation medium, which contained cytosol, mitochondria and pyruvate caused a substantial increase of oxygen consumption. The presence of all of the compounds on which the operation of the PNC depends was necessary to observe this effect. Omission of either aspartate, IMP or GTP resulted in a lack of stimulation of pyruvate oxidation. IMP could be replaced by AMP as the activity of adenylate deaminase in rat skeletal muscle is about 100 times higher than the activities of adenylosuccinate synthetase and adenylosuccinate lyase[6]. At this point it is worth mentioning that under the conditions described in the table about 20 nmoles/min of ammonia were produced when IMP was added and about 160 nmoles/min when IMP was replaced by AMP. Aspartate added without IMP and GTP greatly stimulated pyruvate oxidation due to oxaloacetate formation by aminotransferase activity; thus all the experiments presented in the table were carried out in the presence of aminooxyacetate. It thus appears that under conditions in which adenylosuccinate is formed in the reaction catalyzed by adenylosuccinate lyase, fumarate is delivered to the citric acid cycle at a rate sufficient to maintain a rapid oxidation of pyruvate.

In the rat hindleg muscle, the activity of the rate-limiting enzyme of the PNC is approximately 0.4 μmoles/g fresh weight/min[13]; the 2 other possible anaplerotic processes are less active in skeletal muscle. The maximum activities of extramitochondrial malic enzyme and pyruvate carboxylase have been calculated to be 0.2[14] and 0.03[4] μmoles/g/min[4,14]. Comparing these figures only, one could speculate that the PNC is the most important process responsible for the replenishment of citric acid cycle intermediates in vivo. However, Meyer and Terjung[15] concluded that IMP formation and reamination do not occur simultaneously in some types of skeletal muscle. This might indicate that the PNC does not operate under certain conditions. Nevertheless, under conditions in which both IMP formation and ade-

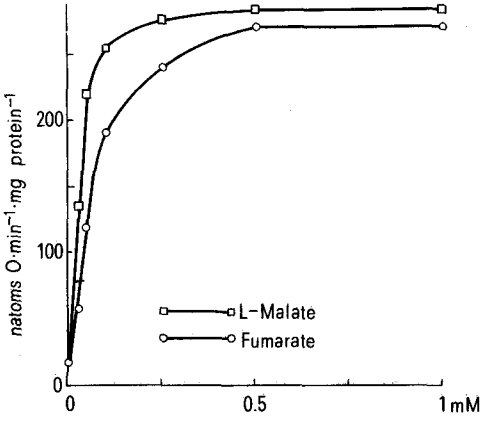

Figure 1. The effect of malate (□—□) or fumarate (○—○) concentration on pyruvate oxidation by rat skeletal muscle mitochondria. The reaction mixture consisted of 25 mM KCl, 50 mM Tris-HCl pH 7.2, 5 mM potassium phosphate buffer pH 7.2, 2 mM $MgCl_2$, 1 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone), 1 mM pyruvate and mitochondria (1.5 mg protein). The malate or fumarate concentrations varied between 0 and 1 mM. The temperature was 30 °C. Total volume 2.5 ml.

The effect of GTP, aspartate and IMP or AMP on the respiration of skeletal muscle mitochondria incubated with pyruvate and cytosol

Conditions	Oxygen uptake ng atoms O × min^{-1} per mg mitochondrial protein
Pyruvate	15
Fumarate	10
Pyruvate + fumarate	265
Pyruvate + GTP	15
Pyruvate + GTP + aspartate	18
Pyruvate + GTP + IMP	15
Pyruvate + IMP + aspartate	18
Pyruvate + aspartate + GTP + IMP	146
Pyruvate + GTP + aspartate + AMP	151

Mitochondria (1.4 mg protein) were incubated at 30 °C with 50 mM Tris-HCl pH 7.2, 5 mM potassium phosphate buffer pH 7.2, 25 mM KCl, 2 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 2 mM aminooxyacetate, 1 μM CCCP, cytosol (4.2 mg protein); 1 mM pyruvate, 1 mM fumarate, 1 mM GTP, 1 mM aspartate, 1 mM IMP, 1 mM AMP as indicated. The final volume was 2.5 ml. Values presented are the means of 3 independent experiments.

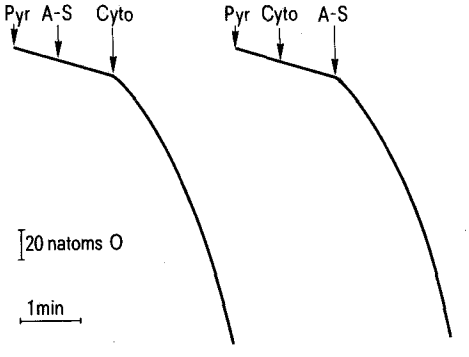

Figure 2. The effect of adenylosuccinate on pyruvate oxidation by rat skeletal muscle mitochondria incubated in the presence of rat skeletal muscle cytosol. Experimental conditions were described in figure 1. Where indicated pyruvate 1 mM (Pyr), adenylosuccinate 0.8 mM (A-S), cytosol 4.2 mg protein (Cyto) were added.

nylosuccinate production are increased, the operation of the PNC must be favored. This would require an activation of AMP deaminase sufficient to produce significant amounts of IMP, without establishing cellular conditions which would inhibit adenylosuccinate synthetase. These circumstances may arise when the rate of ATP hydrolysis is increasing. Hence, the purine nucleotide cycle may be important as anaplerotic process mainly in the working muscle when the ATP level drops. This conclusion is in accordance with the experiments published recently by Aragon and Lowenstein[6].

It should be emphasized that the pyruvate carboxylase activity in rat skeletal muscle is controlled by the ATP/ADP ratio[4]. With a decreasing ATP/ADP ratio, a decrease of the enzyme activity was observed. This suggests that, when ATP level decreases the pyruvate carboxylase activity might be suppressed.

Considering the kinetic properties of malic enzyme from liver[6] and heart mitochondria[4], some authors deny the possibility that the pyruvate carboxylation catalyzed by the malic enzyme could be an important process responsible for the supply of the Krebs cycle intermediates in skeletal muscle. Our results reported recently suggest that pyruvate carboxylation catalyzed by extramitochondrial malic enzyme can increase the level of the citric acid cycle intermediates if the concentration of pyruvate is increased in the tissue. It seems likely, therefore, that when the ATP and pyruvate levels are low in skeletal muscle the PNC is responsible for a substantial part if not for the whole of the increase of the citric acid cycle intermediates.

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Effects of plant sterols on cholesterol concentration in the rat small intestine[1]

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Summary. The effects of feeding single doses of β-sitosterol, campesterol and stigmasterol on cholesterol concentration in the rat small intestine was studied to clarify their roles in cholesterol absorption. The different plant sterols affected free and ester cholesterol concentrations differently in the different intestinal segments suggesting that they have different effects on such intestinal processes as uptake, esterification and possibly synthesis of cholesterol and transport of cholesterol esters out of the mucosal cells into the lymphatics.

β-Sitosterol, campesterol and stigmasterol, the commonly found plant sterols are usually considered together as if they are one single entity. However, in animals and humans the absorption of these sterols are different[2-7] and also both in vivo and in vitro studies have shown that the uptake of these sterols by the rat small intestine is different[8]. It was of interest, therefore, to study the effects of plant sterols individually on cholesterol concentration in the small intestine to clarify their roles on the intestinal processes involved in cholesterol absorption such as uptake and esterification because the well-known hypocholesterolemic effect of 'plant sterols'[5,9,10] may be mediated by their actions on such processes.

Materials and methods. Sitosterol, campesterol and stigmasterol (> 99.5% pure, Applied Science Labs, State College, PA) were used without further purification. Adult male and female Sprague-Dawley rats, each weighing 250-300 g, were fed the commercial pellet diet (Ralston Purina Co., St. Louis, MO) until used. They were fasted for 24 h and under light ether anesthesia, were fed by stomach tube about 2 g olive oil in which the individual plant sterols were dissolved. The composition of the test meals is given in table 1. The control animals were fed the oil only. All animals were fed at about the same time, 9.30 h on the day of the experiment. 4 h later, animals were killed and the small intestine was dissected and collected in ice-cold saline. The intestine was flushed thoroughly with saline, opened longitudinally, again washed thoroughly with saline, and divided into 6 serial segments of about equal length and dried to a constant weight in a heated vacuum

Table 1. Plant sterol concentrations in the test meals

Test meal	Plant sterols, mg/g oil			
	β-Sitosterol	Campesterol	Stigmasterol	Total
Oil	0.27 (65.9)*	0.14 (34.1)	trace**	0.41
Oil + β-sitosterol	10.16 (98.8)	0.12 (1.2)	trace	10.28
Oil + campesterol	0.23 (2.8)	7.98 (97.2)	trace	8.21
Oil + stigmasterol	0.26 (3.5)	0.15 (2.0)	7.08 (94.5)	7.49

* Figures in parentheses are percent of total; ** trace indicates < 0.01 mg/g.